ENHANCED ACCUMULATION OF TREHALOSE IN PLANTS

FIELD OF THE INVENTION

The invention relates to a method for the production of trehalose in plant cells, and plants. The invention is particularly related to a method for increasing the levels of trehalose accumulation in plants by inhibiting the degradation of trehalose by trehalase. The invention further comprises higher plants, preferably *Angiospermae*, and parts thereof, which as a result of such methods, contain relatively high levels of trehalose. The invention further relates to plant cells, plants or parts thereof according to the invention obtained after processing thereof.

STATE OF THE ART

Trehalose is a general name given to D-glucosyl D-glucosides which comprise disaccharides based on two α-,α,ß- and ß,ß-linked glucose molecules. Trehalose, and especially α-trehalose alpha-D-glucopyranosyl(1-1)alpha-D-glucopyranoside is a widespread naturally occurring disaccharide. However, trehalose is not generally found in plants, apart from a few exceptions, such as the plant species *Selaginella lepidophylla (Lycophyta)* and *Myrothamnus flabellifolia*. Apart from these species, trehalose is found in root nodules of the *Leguminosae (Spermatophytae, Angiospermae)*, wherein it is synthesized by bacteroids; the trehalose so produced is capable of diffusing into the root cells. Apart from these accidental occurrences, plant species belonging to the *Spermatophyta* apparently lack the ability to produce and/or accumulate trehalose.

In International patent application WO 95/01446, filed on June 30, 1994 in the name of MOGEN International NV, a method is described for providing plants not naturally capable of producing trehalose with the capacity to do so.

In spite of the absence of trehalose as a substrate in most higher plant species, the occurrence of trehalose-degrading activity has been reported for a considerable number of higher plant species, including those known to lack trehalose. The responsible activity could be attributed to a trehalase enzyme.

Reports suggest that trehalose, when fed to plant shoots grown *in vitro* is toxic or inhibitory to the growth of plant cells (Veluthambi K. *et al.*, 1981, Plant Physiol. 68, 1369-1374). Plant cells producing low trehalase levels were found to be generally more sensitive to the adverse effects of trehalose, than plants exhibiting a higher level of trehalase activity. Trehalose-analogs, such as trehalose-amines were used to inhibit trehalase activity in shoots, making it possible to study the effects of trehalose fed to plant cells. Plant shoots which produce relatively high amounts of trehalase were adversely affected by the addition of trehalase inhibitors. Inhibition of trehalase activity in homogenates of callus and suspension culture of various *Angiospermae* using Validamycin is disclosed by Kendall *et al.*, 1990, Phytochemistry 29, 2525-2582.

It is an object of the present invention to provide plants and plant parts capable of producing and accumulating trehalose.

SUMMARY OF THE INVENTION

The invention provides a process for producing trehalose in plant cells capable of producing trehalase by growing plant cells having the genetic information required for the production of trehalose and trehalase, or cultivating a plant or a part thereof comprising such plant cells, characterised in that said plant cells are grown, or said plant or a part thereof, is cultivated in the presence of a trehalase inhibitor. Preferred plants or plant parts or plant cells have been genetically altered so as to contain a chimeric trehalose phosphate synthase gene in a plant expressible form. According to one embodiment said trehalose phosphate synthase gene comprises an open reading frame encoding trehalose phosphate synthase from *E. coli* in plant expressible form. More preferred is a gene coding for a bipartite enzyme with both trehalose phosphate synthase and trehalose phosphate phosphatase activities.

According to a further aspect of the invention, plants have been genetically altered so as to produce trehalose preferentially in certain tissues or parts, such as (micro-)tubers of potato. According to one embodiment the open reading frame encoding trehalose phosphate synthase from *E. coli* is downstream of the potato patatin promoter, to provide for

preferential expression of the gene in tubers and micro-tubers of *Solanum tuberosum*.

According to another aspect of the invention the plants are cultivated *in vitro*, for example in hydroculture.

According to another preferred embodiment said trehalase inhibitor comprises validamycin A in a form suitable for uptake by said plant cells, preferably in a concentration between 100 nM and 10 mM, preferably between 0.1 and 1 mM, in aqueous solution.

Equally suitable said trehalase inhibition can be formed by transformation of said plant with the antisense gene to a gene encoding the information for trehalase.

Also suitable as trehalase inhibitor is the 86 kD protein from the american cockroach (*Periplaneta americana*). This protein can be administered to a plant in a form suitable for uptake, and also it is possible that the plants are transformed with DNA coding for said protein.

The invention further provides plants and plant parts which accumulate trehalose in an amount above 0.01 % (fresh weight), preferably of a *Solanaceae* species, in particular *Solanum tuberosum* or *Nicotiana tabacum*, in particular a micro-tuber of *Solanum tuberosum* containing trehalose.

The invention also comprises the use of a plant, or plant part, according to the invention for extracting trehalose, as well as the use thereof in a process of forced extraction of water from said plant or plant part. According to yet another embodiment of the invention a chimaeric plant expressible gene is provided, comprising in sequence a transcription initiation region obtainable from a gene, preferentially expressed in a plant part, particularly the patatin gene from *Solanum tuberosum*, a 5'-untranslated leader, an open reading frame encoding a trehalose phosphate synthase activity, and downstream of said open reading frame a transcriptional terminator region.

According to yet another embodiment of the invention a chimaeric plant expressible gene is provided, comprising in sequence a transcription initiation region obtainable from a gene, preferentially expressed in a plant part, particularly the patatin gene from *Solanum tuberosum*, a 5'-untranslated leader, an open reading frame encoding a

trehalase coupled in the antisense orientation, and downstream of said open reading frame a transcriptional terminator region. A preferred plant expressible gene according to the invention is one wherein said transcriptional terminator region is obtainable from the proteinase inhibitor-II gene of *Solanum tuberosum*. The invention also provided vectors and recombinant plant genomes comprising a chimaeric plant expressible gene according to the invention, as well as a plant cell having a recombinant genome, a plant or a part thereof, consisting essentially of cells. A further preferred plant species according to this aspect is *Solanum tuberosum*, and a micro-tuber thereof.

The invention further provides a process for obtaining trehalose, comprising the steps of growing plant cells according to the invention or cultivating a plant according to the invention and extracting trehalose from said plant cells, plants or parts.

The following figures further illustrate the invention.

DESCRIPTION OF THE FIGURES

Figure 1. Schematic representation of binary vector pMOG845.

Figure 2. Schematic representation of multi-copy vector pMOG1192.

Figure 3. Alignments for maximal amino acid similarities of neutral trehalase from *S. cerevisiae* with periplasmatic trehalase from *E. coli*, small intestinal trehalase from rabbit and trehalase from pupal midgut of the silkworm, *Bombyx mori*. Identical residues among all trehalase enzymes are indicated in ***bold italics*** typeface. Conserved regions of the amino acid sequences were aligned to give the best fit. Gap's in the amino acid sequence are represented by dashes.
Positions of degenerated primers based on conserved amino acids are indicated by dashed arrows.

Figure 4. Alignment for maximal amino acid similarity of trehalases derived from *E. coli* (Ecoli2treh ; Ecolitreha), silkworm (Bommotreha), yellow mealworm (Tenmotreha), rabbit (Rabbitreha), *Solanum tuberosum* cv. Kardal (Potatotreha), and *S. cerevisiae* (Yeasttreha). Gap's in the amino

acid sequence are represented by dots.

Figure 5. Trehalase activity in leaf samples of *Nicotiana tabacum* cv. Samsun NN. Non-transgenic control plants are indicated by letters a-l, plants transgenic for pMOG1078 are indicated by numbers.

Figure 6. Trehalose accumulation in microtubers induced on stem segments derived from *Solanum tubersosum* cv. Kardal plants transgenic for both pMOG 845 (patatin driven $TPS_{E.coli}$ expression) and pMOG1027 (35SCaMV antisense-trehalase expression). N indicates the total number of transgenic lines screened. Experiments were performed in duplicate resulting in two values: a and b. ND: not determined.

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention it has been found that the accumulation of an increased level of trehalose in plants and plant parts is feasible. This important finding can be exploited by adapting plant systems to produce and/or accumulate high levels of trehalose at lower cost.

According to one aspect of the invention the accumulation of increased levels of trehalose is achieved by inhibiting endogenous trehalases. Inhibition of trehalases can be performed basically in two ways: by administration of trehalase inhibitors exogenously, and by the production of trehalase inhibitors endogenously, for instance by transforming the plants with DNA sequences coding for trehalase inhibitors.

This inhibition can be equally well applied to plants which are transformed with enzymes which enable the production of trehalose, but also to plants which are able to synthesize trehalose naturally.

According to this first embodiment of the invention, trehalase inhibitors are administered to the plant system exogenously. Examples of trehalase inhibitors that may be used in such a process according to the invention are trehazolin produced in *Micromonospora*, strain SANK 62390 (Ando *et al.*, 1991, J. Antibiot. 44, 1165-1168), validoxylamine A, B, G, D-*gluco*-Dihydrovalidoxylamine A, L-*ido*-Dihydrovalidoxylamin A, Deoxynojirimycin (Kameda *et al.*, 1987, J. Antibiot. 40(4), 563-565), 5-

epi-trehazolin (Trehalostatin) (Kobayashi Y. *et al.*, 1994, J. Antiobiot. 47, 932-938), castanospermin (Salleh H.M. & Honek J.F. March 1990, FEBS 262(2), 359-362) and the 86kD protein from the american cockroach (*Periplaneta americana*) (Hayakawa *et al.*, 1989, J. Biol. Chem. 264(27), 16165-16169).

A preferred trehalase inhibitor according to the invention is validamycin A (1,5,6-trideoxy-3-o-ß-D-glucopyranosyl-5-(hydroxymethyl)-1-[[4,5,6-trihydroxy-3-(hydroxymenthyl)-2-cyclohexen-1-yl]amino]-D-chiro-inositol). Trehalase inhibitors are administered to plants or plant parts, or plant cell cultures, in a form suitable for uptake by the plants, plant parts or cultures. Typically the trehalase inhibitor is in the form of an aqueous solution of between 100 nM and 10 mM of active ingredient, preferably between 0.1 and 1 mM. Aqueous solutions may be applied to plants or plant parts by spraying on leaves, watering, adding it to the medium of a hydroculture, and the like. Another suitable formulation of validamycin is solacol, a commercially available agricultural formulation (Takeda Chem. Indust., Tokyo).

Alternatively, or in addition to using exogenously administered trehalase inhibitors, trehalase inhibitors may be provided by introducing the genetic information coding therefor. One form of such in-built trehalase inhibitor may consist of a genetic construct causing the production of RNA that is sufficiently complementary to endogenous RNA encoding for trehalase to interact with said endogenous transcript, thereby inhibiting the expression of said transcript. This so-called "antisense approach" is well known in the art (*vide* inter alia EP 0 240 208 A and the Examples to inhibit SPS disclosed in WO 95/01446).

A gene coding for trehalase has been isolated from a potato cDNA library and sequenced. The predicted amino acid sequence of trehalase as shown in SEQIDNO:10 is derived from the nucleotide sequence depicted in SEQIDNO: 9. A comparison of this sequence with known non-plant trehalase sequences learns that homology is scant. It is therefor questionable if such trehalase sequences used in an antisense approach are capable of inhibiting trehalase expression *in planta*.

Of course the most preferred embodiment of the invention is obtained by transforming a plant with the antisense trehalase gene which matches exactly with the endogenous trehalase gene. However, sequences

which have a high degree of homology can also be used. Thus, the antisense trehalase gene to be used for the transformation of potato will be dir cted against the nucleotide sequence depicted in SEQIDNO: 9. It is also demonstrated in this application that the potato trehalas sequence can also be used to inhibit trehalase expression in tomato since the potato sequence is highly homologous to the tomato trehalase sequence. Thus, it is envisaged that the potato sequence is usable at least in closely related species, but maybe also in other plants. This is even more the case, considering that it is usually enough to express only part of the homologous gene in the antisense orientation, in order to achieve effective inhibition of expression of the endogenous trehalase (*vide* Van der Krol *et al.*, 1990, Plant Molecular Biology, 14, 457-466). Furthermore, it is shown in this application that the potato trehalase sequence can be used for the detection of homology in other species.

Trehalase gene sequences of other plants can be elucidated using several different strategies. One of the strategies is to use the isolated potato cDNA clone as a probe to screen a cDNA library containing the cDNA of the desired plant species. Positive reacting clones can then be isolated and subcloned into suitable vectors.

A second strategy to identify such genes is by purifying the proteins which are involved in trehalose degradation. An example for such a strategy is the purification of a protein with acid invertase activity from potato (Solanum tuberosum L.) tubers (Burch *et al.*, Phytochemistry, Vol.31, No.6, pp. 1901-1904, 1992). The obtained protein preparation also exhibits trehalose hydrolysing activity. Disaccharide hydrolysing activity of protein preparations obtained after purification steps can be monitored as described by Dahlqvist (Analytical Biochemistry 7, 18-25, 1964).

After purifying the protein(s) with trehalose hydrolysing activity to homogeneity, the N-terminal amino acid sequence or the sequence of internal fragments after protein digestion is determined. These sequences enable the design of oligonucleotide probes which are used in a polymerase chain reaction (PCR) or hybridization experiments to isolate the corresponding mRNAs using standard molecular cloning techniques.

Alternatively, degenerated primers can be designed based on conserved sequences present in trehalase genes isolated from other

species. These primers are used in a PCR strategy to amplify putative trehalase genes. Based on sequence information or Southern blotting, trehalase PCR fragments can be identified and the corresponding cDNA's isolated.

An isolated cDNA encoding a trehalose degrading enzyme is subsequently fused to a promoter sequence in such a way that transcription results in the synthesis of antisense mRNA.

Another form of such an in-built trehalase inhibitor may consist of a genetic construct causing the production of a protein that is able to inhibit trehalase activity in plants. A proteinaceous inhibitor of trehalase has been isolated and purified from the serum of resting adult american cockroaches (*Periplaneta americana*) (Hayakawa et al., *supra*). This protein, of which the sequence partly has been described in said publication, can be made expressable by isolation of the gene coding for the protein, fusion of the gene to a suitable promoter, and transformation of said fused gene into the plant according to standard molecular biological methods.

A promoter may be selected from any gene capable of driving transcription in plant cells.

If trehalose accumulation is only desired in certain plant parts, such as potato (mini-)tubers, the trehalase inhibitory DNA construct (e.*g*. the antisense construct) comprises a promoter fragment that is preferentially expressed in (mini-)tubers, allowing endogenous trehalase levels in the remainder of the plant's cells to be substantially unaffected. Thus, any negative effects of trehalose to neighbouring plant cells due to trehalose diffusion, is counteracted by unaffected endogenous trehalase activity in the remainder of the plant.

In the Example illustrating the invention, wherein trehalose phosphate synthase is produced under the control of the patatin promoter fragment, also the trehalase-inhibitory construct may comprise a promoter fragment of the patatin gene.

Mutatis mutandis if trehalose is to be accumulated in tomato fruit, both a plant expressible trehalose phosphate synthase gene, which is at least expressed in the tomato fruit is to be used, as well as a plant expressible trehalase-inhibitory DNA construct, which should be expressed preferentially in the fruit, and preferably not, or not substantially,

outside the fruit. An example of a promoter fragment that may be used to drive expression of DNA-constructs preferentially in tomato fruit is disclosed in EP 0 409 629 A1. Numerous modifications of this aspect of th invention, that do not depart from the scope of this invention, are readily envisaged by persons having ordinary skill in the art to which this invention pertains.

An alternative method to block the synthesis of undesired enzymatic activity such as caused by endogenous trehalase is the introduction into the genome of the plant host of an additional copy of said endogenous trehalase gene. It is often observed that the presence of a transgene copy of an endogenous gene silences the expression of both the endogenous gene and the transgene (EP 0 465 572 A1).

According to one embodiment of the invention accumulation of trehalose is brought about in plants wherein the capacity of producing trehalose has been introduced by introduction of a plant expressible gene construct encoding trehalose phosphate synthase (TPS), see for instance WO 95/06126.

Any trehalose phosphate synthase gene under the control of regulatory elements necessary for expression of DNA in plant cells, either specifically or constitutively, may be used, as long as it is capable of producing active trehalose phosphate synthase activity. Most preferred are the trehalose phosphate synthase genes which also harbour a coding sequence for trehalose phosphate phosphatase activity, the so called bipartite enzymes. Such a gene, formerly only known to exist in yeast (see *e.g.* WO 93/17093), can also been found in most plants. This application describes the elucidation of such a gene from the sunflower *Helianthus annuus*, while also evidence is given for the existence of a homologous gene in *Nicotiana tabacum*. It is believed that the use of a bipartite enzyme enhances the production of trehalose because it enables completion of the metabolic pathway from UDP-glucose and glucose-6-phosphate into trehalose at one and the same site. Hence, the two-step synthesis is simplified into a one-step reaction, thereby increasing reaction speed and, subsequently, trehalose yield.

As genes involved in trehalose synthesis, especially genes coding for bipartite enzymes, become available from other sources these can be used in a similar way to obtain a plant expressible trehalose

synthesizing gene according to the invention.

Sources for isolating trehalose synthesizing activities include microorganisms (*e.g.* bacteria, yeast, fungi), but these genes can also be found in plants and animals.

The invention also encompasses nucleic acid sequences which have been obtained by modifying the nucleic acid sequence encoding enzymes active in the synthesis of trehalose by mutating one or more codons so that it results in amino acid changes in the encoded protein, as long as mutation of the amino acid sequence does not entirely abolish trehalose synthesizing activity.

According to another embodiment of the invention, plants are genetically altered to produce and accumulate trehalose in specific parts of the plant, which were selected on the basis of considerations such as substrate availability for the enzyme, insensitivity of the plant part to any putative adverse effects of trehalose on plant cell functioning, and the like. A preferred site for trehalose synthesising enzyme expression are starch storage parts of plants. In particular potato tubers are considered to be suitable plant parts. A preferred promoter to achieve selective enzyme expression in microtubers and tubers of potato is obtainable from the region upstream of the open reading frame of the patatin gene of potato (*Solanum tuberosum*).

Plants provide with a gene coding for trehalose phosphate synthase only may be further modified by introducing additional genes that encode phosphatases that are capable of the conversion of trehalose phosphate into trehalose. At least in potato tubers or micro-tubers, potato leaves and tobacco leaves and roots, endogenous phosphatase activity appears to be present, so that the introduction of a trehalose phosphate phosphatase (TPP) gene is not an absolute requirement.

Preferred plant hosts among the *Spermatophyta* are the *Angiospermae*, notably the *Dicotyledoneae*, comprising *inter alia* the *Solanaceae* as a representative family, and the *Monocotyledoneae*, comprising *inter alia* the *Gramineae* as a representative family. Suitable host plants, as defined in the context of the present invention include plants (as well as parts and cells of said plants) and their progeny which have been genetically modified using recombinant DNA techniques to cause or enhance production of trehalose in the desired plant or plant organ; these plants

may be used directly (*e.g.* the plant species which produce edible parts) in processing or the trehalose may be extracted and/or purified from said host. Crops with edible parts according to the invention include those which have flowers such as cauliflower (*Brassica oleracea*), artichoke (*Cynara scolymus*), fruits such as apple (*Malus, e.g. domesticus*), banana (*Musa, e.g. acuminata*), berries (such as the currant, *Ribes, e.g. rubrum*), cherries (such as the sweet cherry, *Prunus, e.g. avium*), cucumber (*Cucumis, e.g. sativus*), grape (*Vitis, e.g. vinifera*), lemon (*Citrus limon*), melon (*Cucumis melo*), nuts (such as the walnut, *Juglans, e.g. regia;* peanut, *Arachis hypogeae*), orange (*Citrus, e.g. maxima*), peach (*Prunus, e.g. persica*), pear (*Pyra, e.g. communis*), pepper (*Solanum, e.g. capsicum*), plum (*Prunus, e.g. domestica*), strawberry (*Fragaria, e.g. moschata*), tomato (*Lycopersicon, e.g. esculentum*), leafs, such as alfalfa (*Medicago sativa*), cabbages (such as *Brassica oleracea*), endive (*Cichoreum, e.g. endivia*), leek (*Allium porrum*), lettuce (*Lactuca sativa*), spinach (*Spinaciaoleraceae*), tobacco (*Nicotiana tabacum*), roots, such as arrowroot (*Maranta arundinacea*), beet (*Beta vulgaris*), carrot (*Daucus carota*), cassava (*Manihot esculenta*), turnip (*Brassica rapa*), radish (*Raphanus sativus*), yam (*Dioscorea esculenta*), sweet potato (*Ipomoea batatas*) and seeds, such as bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), soybean (*Glycin max*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), corn (*Zea mays*), rice (*Oryza sativa*), tubers, such as kohlrabi (*Brassica oleraceae*), potato (*Solanum tuberosum*), and the like. The edible parts may be conserved by drying in the presence of enhanced trehalose levels produced therein due to the presence of a plant expressible trehalose phosphate synthase gene.

The method of introducing the plant expressible gene coding for a trehalose-synthesizing enzyme, or any other sense or antisense gene into a recipient plant cell is not crucial, as long as the gene is expressed in said plant cell. The use of *Agrobacterium tumefaciens or Agrobacterium rhizogenes* - mediated transformation is preferred, but other procedures are available for the introduction of DNA into plant cells. Examples are transformation of protoplasts using the calcium/polyethylene glycol method, electroporation, microinjection and DNA-coated particle bombardment (Potrykus, 1990, Bio/Technol. 8, 535-542). Also combinations of *Agrobacterium* and coated particle bombardment may be used. Also

transformation protocols involving other living vectors than *Agrobacterium* may be used, such as viral vectors (*e.g.* from the Cauliflower Mosaic Virus (CaMV) and or combinations of *Agrobacterium* and viral vectors, a procedure referred to as agroinfection (Grimsley N. *et al.*, 8 January 1987, Nature 325, 177-179). After selection and/or screening, the protoplasts, cells or plant parts that have been transformed are regenerated into whole plants, using methods known in the art (Horsch *et al.*, 1985, Science 225, 1229-1231).

The development of reproducible tissue culture systems for monocotyledonous crops, together with methods for introduction of genetic material into plant cells has facilitated transformation. Presently, preferred methods for transformation of monocot species are transformation with supervirulent *Agrobacterium*-strains, microprojectile bombardment of explants or suspension cells, and direct DNA uptake or electroporation (Shimamoto, *et al.*, 1989, Nature 338, 274-276). *Agrobacterium*-mediated transformation is functioning very well in rice (WO 94/00977). Transgenic maize plants have been obtained by introducing the *Streptomyces hygroscopicus bar*-gene, which encodes phosphinothricin acetyltransferase (an enzyme which inactivates the herbicide phosphinothricin), into embryogenic cells of a maize suspension culture by microprojectile bombardment (Gordon-Kamm, 1990, Plant Cell, 2, 603-618). The introduction of genetic material into aleurone protoplasts of other monocot crops such as wheat and barley has been reported (Lee, 1989, Plant Mol. Biol. 13, 21-30). Wheat plants have been regenerated from embryogenic suspension culture by selecting only the aged compact and nodular embryogenic callus tissues for the establishment of the embryogenic suspension cultures (Vasil, 1990 Bio/Technol. 8, 429-434).

Suitable DNA sequences for control of expression of the plant expressible genes (including marker genes), such as transcriptional initiation regions, enhancers, non-transcribed leaders and the like, may be derived from any gene that is expressed in a plant cell. Also intended are hybrid promoters combining functional portions of various promoters, or synthetic equivalents thereof. Apart from constitutive promoters, inducible promoters, or promoters otherwise regulated in their expression pattern, *e.g.* developmentally or cell-type specific, may be used to control expression of the plant expressible genes according to the

invention as long as they ar expressed in plant parts that contain substrate for TPS.

To select or screen for transform d cells, it is preferred to include a marker gene linked to the plant expressible gene according to the invention to be transferred to a plant cell. The choice of a suitable marker gene in plant transformation is well within the scope of the average skilled worker; some examples of routinely used marker genes are the neomycin phosphotransferase genes conferring resistance to kanamycin (EP-B 131 623), the glutathion-S-transferase gene from rat liver conferring resistance to glutathione derived herbicides (EP-A 256 223), glutamine synthetase conferring upon overexpression resistance to glutamine synthetase inhibitors such as phosphinothricin (WO87/05327), the acetyl transferase gene from *Streptomyces viridochromogenes* conferring resistance to the selective agent phosphinothricin (EP-A 275 957), the gene encoding a 5-enolshikimate-3- phosphate synthase (EPSPS) conferring tolerance to N-phosphonomethylglycine, the *bar* gene conferring resistance against Bialaphos (*e.g.* WO 91/02071) and the like. The actual choice of the marker is not crucial as long as it is functional (*i.e.* selective) in combination with the plant cells of choice.

The marker gene and the gene of interest do not have to be linked, since co-transformation of unlinked genes (U.S. Patent 4,399,216) is also an efficient process in plant transformation.

Preferred plant material for transformation, especially for dicotyledonous crops are leaf-discs which can be readily transformed and have good regenerative capability (Horsch R.B. *et al.*, (1985) Science 227, 1229-1231).

It is immaterial to the invention how the presence of two or more genes in the same plant is effected. This can *inter alia* done be achieved by one of the following methods:

(a) transformation of the plant line with a multigene construct containing more than one gene to be introduced,

(b) co-transforming different constructs to the same plant line simultaneously,

(c) subsequent rounds of transformation of the same plant with the genes to be introduced,

(d) crossing two plants each of which contains a different gene to be

introduced into the same plant, or

(e) combinations thereof.

The field of application of the invention lies both in agriculture and horticulture, for instance due to improved properties of the modified plants as such (*e.g.* stress tolerance, such as cold tolerance, and preferably drought resistance, and increase in post-harvest quality and shelf-life of plants and plant products), as well as in any form of industry where trehalose is or will be applied in a process of forced water extraction, such as drying or freeze drying. Trehalose can be used or sold as such, for instance in purified form or in admixtures, or in the form of a plant product, such as a tuber, a fruit, a flower containing the trehalose, either in native state or in (partially) dehydrated form, and the like. Plant parts harbouring (increased levels of) trehalose phosphate or trehalose may be used or sold as such or processed without the need to add trehalose.

Also trehalose can be extracted and/or purified from the plants or plant parts producing it and subsequently used in an industrial process. In the food industries trehalose can be employed by adding trehalose to foods before drying. Drying of foods is an important method of preservation. Trehalose seems especially useful to conserve food products through conventional air-drying, and to allow for fast reconstitution upon addition of water of a high quality product (Roser *et al.*, July 1991, Trends in Food Science and Technology, pp. 166-169). The benefits include retention of natural flavors/fragrances, taste of fresh product, and nutritional value (proteins and vitamins). It has been shown that trehalose has the ability to stabilize proteins e.g. vaccines, enzymes and membranes, and to form a chemically inert, stable glass. The low water activity of such thoroughly dried food products prevents chemical reactions, that could cause spoilage.

Field crops like corn, cassava, potato, sugar beet and sugarcane have since long been used as a natural source for bulk carbohydrate production (starches and sucrose). The production of trehalose in such crops, facilitated by genetic engineering of the trehalose-biosynthetic pathway into these plant species, would allow the exploitation of such engineered crops for trehalose production.

Trehalose is also used in drying or storage of biological macromolecules, such as peptides, enzymes, polynucleotides and the like.

All references cited in this specification are indicative of the level of skill in the art to which the invention pertains. All publications, whether patents or otherwise, referred to previously or later in this specification are herein incorporated by reference as if each of them was individually incorporated by reference. In particular WO 95/01446, cited herein, describing the production of trehalose in higher plants by genetic manipulation is herein incorporated by reference.

The Examples given below illustrate the invention and are in no way intended to indicate the limits of the scope of the invention.

Experimental

DNA manipulations

All DNA procedures (DNA isolation from *E.coli*, restriction, ligation, transformation, etc.) are performed according to standard protocols (Sambrook *et al.* (1989) Molecular Cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, CSH, New York).

Strains

In all examples E.coli K-12 strain DH5α is used for cloning. The *Agrobacterium tumefaciens* strains used for plant transformation experiments are EHA 105 and MOG 101 (Hood *et al.* 1993, Trans. Research 2, 208-218)

Isolation of a patatin promoter/construction of pMOG546

A patatin promoter fragment is isolated from chromosomal DNA of *Solanum tuberosum* cv. Bintje using the polymerase chain reaction. A set of oligonucleotides, complementary to the sequence of the upstream region of the λpat21 patatin gene (Bevan, M., Barker, R., Goldsbrough, A., Jarvis, M., Kavanagh, T. and Iturriaga, G. (1986) Nucleic Acids Res. 14: 5564-5566), is synthesized consisting of the following sequences:

5′ AAG CTT ATG TTG CCA TAT AGA GTA G 3′	PatB33.2	(SEQIDNO:3)
5′ GTA GTT GCC ATG GTG CAA ATG TTC 3′	PatATG.2	(SEQIDNO:4)

Th se primers are used to PCR amplify a DNA fragment of 1123bp, using chromosomal DNA isolated from potato cv. Bintje as a template. The amplified fragment shows a high degree of similarity to the λpat21 patatin sequence and is cloned using EcoRI linkers into a pUC18 vector resulting in plasmid pMOG546.

Construction of pMOG 799

pMOG 799 harbours the TPS gene from *E. coli* under control of the double enhanced 35S Cauliflower Mosaic promoter. The construction of this binary vector is described in detail in International patent application WO 95/01446, incorporated herein by reference.

Construction of pMOG845.

Plasmid pMOG546 containing the patatin promoter is digested with NcoI-KpnI, incubated with *E. coli* DNA polymerase I in the presence of dATP and dCTP thereby destroying the NcoI and KpnI site and subsequently relegated. From the resulting vector a 1.1kb EcoRI-SmaI fragment containing the patatin promoter is isolated and cloned into pMOG798 (described in detail in WO 95/01446) linearized with SmaI-EcoRI consequently exchanging the 35S CaMV promoter for the patatin promoter. The resulting vector is linearized with HindIII and ligated with the following oligonucleotide duplex:

	(HindIII) PstI	KpnI HindIII		
5′	AGCT CTGCAG	TGA GGTACC A	3′	TCV 11 (SEQIDNO:5)
3′	GACGTC ACT	CCATGG TTCGA	5′	TCV 12 (SEQIDNO:6)

After checking the orientation of the introduced oligonucleotide duplex, the resulting vector is linearized with PstI-HindIII followed by the insertion of a 950bp PstI-HindIII fragment harbouring the potato proteinase inhibitor II terminator (PotPiII) (An, G., Mitra, A., Choi, H.K., Costa, M.A., An, K., Thornburg, R. W. and Ryan, C.A. (1989) The Plant Cell 1: 115-122). The PotPiII terminator is isolated by PCR amplification using chromosomal DNA isolated from potato cv. Desiree as a template and the following set of oligonucleotides:

5′	GTACCCTGCAGTGTGACCCTAGAC	3′	TCV 15	(SEQIDNO:7)
5′	TCGATTCATAGAAGCTTAGAT	3′	TCV 16	(SEQIDNO:8)

The TPS expression cassette is subsequently cloned as a EcoRI-HindIII fragment into the binary vector pMOG402 resulting in pMOG845 (fig. 1). A sample of *E.coli* Dhα strain, harbouring pMOG845 has been deposited at the Centraal Bureau voor Schimmelcultures, Oosterstraat 1, P.O. Box 273, 3740 AG Baarn, The Netherlands, on January 4, 1995; the Accession Number given by the International Depositary Institution is CBS 101.95.

Triparental matings

The binary vectors are mobilized in triparental matings with the *E. coli* strain HB101 containing plasmid pRK2013 (Ditta G., Stanfield, S., Corbin, D., and Helinski, D.R. *et al.* (1980) Proc. Natl. Acad. Sci. USA 77, 7347) into *Agrobacterium tumefaciens* strain MOG101 or EHA105 and used for transformation.

Transformation of tobacco (*Nicotiana tabacum* SR1)

Tobacco is transformed by cocultivation of plant tissue with *Agrobacterium tumefaciens* strain MOG101 containing the binary vector of interest as described. Transformation is carried out using cocultivation of tobacco (*Nicotiana tabacum* SR1) leaf disks as described by Horsch *et al.* 1985, Science 227, 1229-1231. Transgenic plants are regenerated from shoots that grow on selection medium containing kanamycin, rooted and transferred to soil.

Transformation of potato tuber discs

Potato (*Solanum tuberosum* cv. Kardal) is transformed with the *Agrobacterium* strain EHA 105 containing the binary vector of interest. The basic culture medium is MS30R3 medium consisting of MS salts (Murashige, T. and Skoog, F. (1962) Physiol. Plan. 14, 473), R3 vitamins (Ooms *et al.* (1987) Theor. Appl. Genet. 73, 744), 30 g/l sucrose, 0.5 g/l MES with final pH 5.8 (adjusted with KOH) solidified when necessary with 8 g/l Daichin agar. Tubers of *Solanum tuberosum* cv. Kardal are peel d and surface sterilized by burning them in 96% ethanol for 5 seconds.

Extinguish the flames in sterile water and cut slices of approximately 2 mm thickness. Disks are cut with a bore from the vascular tissue and incubated for 20 minutes in MS30R3 medium containing 1-5 x10^8 bacteria/ml of *Agrobacterium* EHA 105 containing the binary vector. Wash the tuber discs with MS30R3 medium and transfer them to solidified postculture medium (PM). PM consists of M30R3 medium supplemented with 3.5 mg/l zeatin riboside and 0.03 mg/l indole acetic acid (IAA). After two days, discs were transferred to fresh PM medium with 200 mg/l cefotaxim and 100 mg/l vancomycin. Three days later, the tuber discs are transferred to shoot induction medium (SIM) which consists of PM medium with 250 mg/l carbenicillin and 100 mg/l kanamycin. After 4-8 weeks, shoots emerging from the discs are excised and placed on rooting medium (MS30R3-medium with 100 mg/l cefotaxim, 50 mg/l vancomycin and 50 mg/l kanamycin). The shoots are propagated axenically by meristem cuttings.

Potato stem-segment transformation protocol.

Potato transformation experiments using stem-internodes were performed in a similar way as described by Newell C.A. *et al.*, Plant Cell Reports 10: 30-34, 1990.

Induction of micro-tubers

Stem segments of *in vitro* potato plants harbouring an auxiliary meristem are transferred to micro-tuber inducing medium. Micro-tuber inducing medium contains 1 X MS-salts supplemented with R3 vitamins, 0.5 g/l MES (final pH= 5.8, adjusted with KOH) and solidified with 8 g/l Daishin agar, 60 g/l sucrose and 2.5 mg/l kinetin. After 3 to 5 weeks of growth in the dark at 24°C, micro-tubers are formed.

Trehalose assay

Trehalose was determined quantitatively by anion exchange chromatography with pulsed amperometric detection. Extracts were prepared by adding 1 ml boiling water to 1 g frozen material which was subsequently heated for 15′ at 100°C. Samples (25 µl) were analyzed on a Dionex DX-300 liquid chromatograph equipped with a 4 x 250 mm Dionex 35391 carbopac PA-1 column and a 4 x 50 mm Dionex 43096 carbopac PA-1 precolumn. Elution was with 100 mM NaOH at 1 ml/min. Sugars were detected with a pulsed

amperometric d t ctor (Dion x, PAD-2). Commercially available tr halose (Sigma) was used as a standard.

Isolation of Validamycin A

Validamycin A is isolated from Solacol, a commercial agricultural formulation (Takeda Chem. Indust., Tokyo) as described by Kendall *et al.* (1990) Phytochemistry, Vol. 29, No. 8, pp. 2525-2528. The procedure involves ion exchange chromatography (QAE-Sephadex A-25 (Pharmacia), bed vol. 10 ml, equilibration buffer 0.2 mM Na-Pi pH 7) from a 3% agricultural formulation of Solacol. Loading 1 ml of Solacol on the column and eluting with water in 7 fractions, practically all Validamycin is recovered in fraction 4.

Based on a 100% recovery, using this procedure, the concentration of Validamycin A was adjusted to 110^{-3} M in MS-buffer, for use in trehalose accumulation tests.

Alternatively, Validamycin A and B may be purified directly from *Streptomyces hygroscopicus* var. *limoneus*, as described by Iwasa T. *et al.*, 1971, in The Journal of Antibiotics 24(2), 119-123, the content of which is incorporated herein by reference.

Construction of pMOG1027

pMOG1027 harbours the trehalase gene from *Solanum tuberosum* cv. Kardal in the reversed orientation under control of the double enhanced 35S Cauliflower Mosaic promoter. The construction of this vector is very similar to the construction of pMOG799 and can be performed by any person skilled in the art. After mobilization of this binary vector by triparental mating to *Agrobacterium*, this strain can be used to transform plant cells and to generate transgenic plants having reduced levels of trehalase activity.

Construction of pMOG1028

pMOG1028 harbours the trehalase gene from *Solanum tuberosum* cv. Kardal in the reversed orientation under control of the tuber specific patatin promoter. The construction of this vector is very similar to the construction of pMOG845 and can be performed by any person skilled in the

art. After mobilization of this binary vector by triparental mating to *Agrobacterium*, this strain can be used in potato transformation experiments to generate transgenic plants having reduced levels of trehalase activity in tuber-tissue.

Construction of pMOG 1078

To facilitate the construction of a binary expression cassette harbouring the trehalase cDNA clone in the "sense" orientation under control of the double enhanced 35S CaMV promoter, two HindIII sites were removed from the trehalase cDNA coding region (without changing the amino acid sequence) by PCR based point-mutations. In this way, a BamHI fragment was engineered that contained the complete trehalase open reading frame. This fragment was subsequently used for cloning in the binary vector pMOG800 behind the constitutive de35S CaMV promoter yielding pMOG1078. pMOG800 is derived from pMOG402; the KpnI site in the polylinker has been restored. pMOG402 is derived of pMOG23 (described in WO 95/01446) and harbours a restored neomycin phosphotransferase gene (Yenofsky R.L., Fine M., Pellow J.W., Proc Natl Acad Sci USA **87**: 3435-3439, 1990).

EXAMPLE 1

Trehalose production in tobacco plants transformed with pMOG799

Tobacco leaf discs are transformed with the binary vector pMOG799 using Agrobacterium tumefaciens. Transgenic shoots are selected on kanamycin. Transgenic plants are transferred to the greenhouse to flower and set seed after selfing (S1). Seeds of these transgenic plants are surface sterilised and germinated *in vitro* on medium with Kanamycin. Kanamycin resistant seedlings and wild-type tobacco plants are transferred to MS-medium supplemented with 10^{-3} M Validamycin A. As a control, transgenic seedlings and wild-type plants are transferred to medium without Validamycin A. Analysis of leaves and roots of plants grown on Validamycin A shows elevated levels of trehalose compared to the control plants (Table 1). No trehalose was detected in wild-type tobacco plants.

Table 1

	with Validamycin A		without Validamycin A	
	leaf	roots	leaf	roots
pMOG799.1	0.0081	0.0044	-	0.003
pMOG799.13	0.0110	0.0080	-	-
pMOG799.31	0.0008	0.0088	-	-
Wild-type SR1	-	-	-	-

EXAMPLE 2

Trehalose production in potato micro-tubers transformed with pMOG845

Potato *Solanum tuberosum* cv. Kardal tuber discs are transformed with *Agrobacterium tumefaciens* EHA105 harbouring the binary vector pMOG845. Transgenic shoots are selected on kanamycin. Micro-tubers (m-tubers) are induced on stem segments of transgenic and wild-type plants cultured on m-tuber inducing medium supplemented with 10^{-3} M Validamycin A. As a control, m-tubers are induced on medium without Validamycin A. M-tubers induced on medium with Validamycin A showed elevated levels of trehalose in comparison with m-tubers grown on medium without Validamycin A (Table 2). No trehalose was detected in wild-type m-tubers.

Table 2.

	Trehalose (% fresh weight)	
	+Validamycin A	-Validamycin A
845-2	0.016	-
845-4	-	-
845-8	0.051	-
845-13	0.005	-
845-22	0.121	-
845-25	0.002	-
wT Kardal	-	-

EXAMPLE 3

Trehalose production in hydrocultures of tobacco plants transformed with pMOG799

Seeds (S1) of selfed tobacco plants transformed with the binary vector pMOG799 are surface sterilised and germinated *in vitro* on MS20MS medium

containing 50 µg/ml Kanamycin. Kanamycin resistant seedlings are transferred to soil and grown in a growth chamber (temp. 23°C, 16 hours of light/day). After four weeks, seedlings were transferred to hydrocultures with ASEF clay beads with approximately 450 ml of medium. The medium contains 40 g/l Solacol dissolved in nano-water buffered with 0.5 g/l MES to adjust to pH 6.0 which is sieved through a filter to remove solid particles. Essential salts are supplemented by adding POKON™ (1.5 ml/l). The following antibiotics are added to prevent growth of micro-organisms: 500µg/ml Carbenicillin, 40µg/ml Nystatin and 100µg/ml Vancomycin. As a control, transgenic seedlings and wild-type plants are transferred to medium without Solacol. Analysis of leaves of plants grown on Solacol shows elevated levels of trehalose compared to the control plants (Table 3). No trehalose was detected in wild-type tobacco plants.

Table 3

	Solacol	Trehalose (%w/w)
pMOG 799.1-1	+	0.008
pMOG 799.1-2	+	0.004
pMOG 799.1-3	-	-
pMOG 799.1-4	-	-
pMOG 799.1-5	+	0.008
pMOG 799.1-6	-	-
pMOG 799.1-7	+	0.005
pMOG 799.1-8	-	-
pMOG 799.1-9	-	-
pMOG 799.1-10	+	0.007
Wild-type SR1-1	-	-
Wild-type SR1-2	+	-
Wild-type SR1-3	-	-
Wild-type SR1-4	+	-

Example 4

Cloning of a full length cDNA encoding trehalase from potato tuber

Using the amino acid sequence of the conserved regions of known trehalase genes (*E.coli*, Yeast, Rabbit, *B. mori*) (fig. 3), four degenerated primers were designed:

GG(C/T)GGI(C/A)G(C/A)TT(CGT/TAA)IGA(GT/AG)(A/T/C)TA(TTAT/CGGC)TGGGAC Tase24 (SEQIDNO:11)

GTICCIGGIGGICGITT(TAA/CGT)IGA(GT/AG)T Tase25 (SEQIDNO:12)

GGIGG(T/C)TGI(GA/CT)ICGI(TG/CA)IAG(A/G)TA(A/G)TA Tase26 (SEQIDNO:13)

I(C/G/A)C(AT/TI/GC)TTI(A/G)CCATCCAAICCITC Tase27 (SEQIDNO:14)

Combinations of these primers in PCR experiments with genomic DNA and cDNA from *S. tuberosum* cv. Kardal leaf and tuber material respectively as template, resulted in several fragments of the expected length. A number of 190 bp. fragments obtained with the primer combination Tase24 and Tase 26 were subcloned into a pGEM T vector and sequenced. Several of the clones analyzed showed homology with known trehalase sequences. To exclude the isolation of non-plant derived trehalase sequences, Southern blot analysis was performed with gDNA from potato cv. Kardal. A number of clones isolated did not cross-hybridize with Kardal genomic DNA and were discarded. Two isolated clones were identical, gTase15.4 derived from a genomic PCR experiment and cTase5.2 derived from a PCR on cDNA, both showing hybridization in Southern blot analysis. One single hybridizing band was detected (EcoRI 1.5 Kb, HindIII 3 Kb and BamHI larger than 12 Kb) suggesting the presence of only one copy of the isolated PCR fragment.

A cDNA library was constructed out of poly A^+ RNA from potato tubers (cv. Kardal) using a Stratagene cDNA synthesis kit and the vector Lambda ZAPII. Recombinant phages (500.000) were screened with the radiolabeled cTase5.2 PCR fragment resulting in the identification of 3 positive clones. After purification, two clones were characterised with restriction enzymes revealing inserts of 2.15 and 2.3 kb respectively. Their nucleotide sequence was 100% identical. The nucleic acid sequenc of one of these trehalase cDNA clones from *Solanum tuberosum* including

its open reading frame is depicted in SEQIDNO:9, while the aminoacid sequence derived from this nucleic acid sequence is shown in SEQIDNO:10. A plasmid harbouring an insert comprising the genetic information coding for trehalase has been deposited under no. CBS 804.95 with the Centraal Bureau voor Schimmelcultures, Oosterstraat 1, P.O. Box 273, 3740 AG Baarn, the Netherlands on December 8, 1995.

EXAMPLE 5

Homology between the trehalase gene from potato with other Solanaceae

Genomic DNA was isolated from tomato (Lycopersicon esculentum cv. Money maker), tobacco (Nicotiana tabacum cv. Petit havanna, SR1) and potato (Solanum tuberosum cv. Kardal), and subsequently digested with the restriction enzymes BamHI, BglII, NcoI, SpeI, AccI, HindIII and EcoRI. After gel-electrophoresis and Southern blotting, a [^{32}P]-alpha dCTP labelled trehalase potato cDNA probe was hybridized to the blot. Hybridization signals of almost similar strength were observed in the lanes with potato and tomato genomic DNA indicating a high degree of identity. Only a weak hybridization signal was observed in the lanes harbouring tobacco genomic DNA indicating a low degree of identity. A similar strategy can be used to identify trehalase genes from other crops and to select for crops were trehalase activity can be eliminated, via the anti-sense expression strategy, using a heterologous trehalase cDNA clone with sufficient homology. Alternatively, a homologous trehalase cDNA clone can be isolated and used in the anti-sense expression strategy.

EXAMPLE 6

Overexpression of a potato trehalase cDNA in *Nicotiana tabacum*

Tobacco leaf discs are transformed with the binary vector pMOG1078 using Agrobacterium tumefaciens. Transgenic shoots are selected on kanamycin and transferred to the greenhouse. Trehalase activity was determined in leaf samples of 26 transgenic and 12 non-transgenic control plants (Fig. 5). Trehalase activity up to ca. 17 µg trehalose/h/µg protein was measured compared to ca. 1 µg trehalose/h/µg protein for non-transgenic controls. This clearly confirms the identity of the potato trehalase cDNA.

EXAMPLE 7

Transformation of pMOG845 transgenic potato plants with pMOG1027

In order to super-transform pMOG845 transgenic potato lines with an anti-sense trehalase construct (pMOG1027), stem segments were cut from in vitro cultured potato shoots transgenic for pMOG845. Three parent lines were selected, pMOG845/11, /22 and /28 that revealed to accumulate trehalose in microtubers when grown on validamycin A. The stem segments were transformed with the binary vector pMOG1027 using Agrobacterium tumefaciens. Supertransformants were selected on Hygromycin and grown in vitro.

EXAMPLE 8

Trehalose production in tubers of potato plants transgenic for pMOG845 and pMOG1027

Microtubers were induced on explants of the pMOG845 transgenic potato plants supertransformed with pMOG1027 using medium without the trehalase inhibitor validamycin A. The accumulation of trehalose, up to 0.75 mg.g-1 fresh weight, was noted in the supertransformed lines proving the reduced trehalase activity in these lines using the anti-sense trehalase expression strategy (Fig. 6).

EXAMPLE 9

Isolation of a bipartite TPS/TPP gene from *Helianthus annuus*

To isolate a bipartite clone from H. annuus, a PCR amplification experiment was set up using two degenerate primers, TPS-deg2 and TPS-deg5. This primerset was used in combination with cDNA constructed on H. annuus leaf RNA as a template. A DNA fragment of approximately 650 bp. was amplified having a high similarity on amino acid level when compared to tps coding regions from E. coli and yeast. Based on its nucleotide sequence, homologous primers were designed and used in a Marathon RACE protocol (Clontech) to isolate the 5′ and 3′ parts of corresponding tps cDNA's. Using primercombinations SUNGSP1(or 2)/AP1 in RACE PCR, no bands were observed whereas nested PCR with NSUNGSP1(or2)/AP2 resulted in several DNA fragments. Some of these fragments hybridized with a 32P labelled Sunflower tps fragment after Southern blotting. Two fragments of

circa 1.2 kb and 1.7 kb, corresponding respectively to the 5′ and 3′ part, were isolated from gel, subcloned and sequenced. The nucleotide sequence revealed a clear homology with known tps and tpp sequences indicating the bipartite nature of the isolated cDNA (SEQ ID NO 1). Using a unique XmaI site present in both fragments, a complete TPS/TPP bipartite coding region was obtained and subcloned in pGEM-T (Promega) yielding pMOG1192 (Fig. 2).

TPSdeg2:	tig git kit tyy tic aya yic cit tyc c	(SEQIDNO: 23)
TPSdeg5:	gyi aci arr ttc ati ccr tci c	(SEQIDNO: 27)
SUNGSP1:	cga aac ggg ccc atc aat ta	(SEQIDNO: 15)
SUNGSP2:	tcg atg aga tca atg ccg ag	(SEQIDNO: 16)
AP1 (Clontech):	cca tcc taa tac gac tca cta tag ggc	(SEQIDNO: 17)
NSUNGSP1:	cac aac agg ctg gta tcc cg	(SEQIDNO: 18)
NSUNGSP2:	caa taa cga act ggg aag cc	(SEQIDNO: 19)
AP2 (Clontech):	act cac tat agg gct cga gcg gc	(SEQIDNO: 20)

EXAMPLE 10

Isolation of a bipartite TPS/TPP gene from *Nicotiana tabacum*

Another strategy to isolate bipartite TPS/TPP genes from plants or other organisms involved the combined use of TPS and TPP primers in a single PCR reaction. As an example, a PCR was performed using cDNA generated on tobacco leaf total RNA and the primerset TPSdeg1 and TRE-TPP-16. Nested PCR, using the amplification mix of the first reaction as template, with TPSdeg2 and TRE-TPP-15 resulted in a DNA fragment of ca. 1.5 kb. Nested PCR of the original amplification mix with TPSdeg2 and TRE-TPP-10 yielded a DNA fragment of ca.1.2 kb.

Initial amplification using primer combination TPSdeg1 and TRE-TPP-6 followed by a nested PCR using primer combination TPSdeg2 and TRE-TPP-15 yielded a DNA fragment of ca. 1.5 kb.

Based on sequence analysis, the 1.2 kb and 1.5 kb amplified DNA fragments displayed a high degree of identity to TPS and TPP coding regions indicating that they encode a bipartite TPS/TPP proteins.

TPSdeg1:	GAY ITI ATI TGG RTI CAY GAY TAY CA	(SEQIDNO: 21)
TRE-TPP-16:	CCI ACI GTR CAI GCR AAI AC	(SEQIDNO: 22)
TPSdeg2:	TIG GIT KIT TYY TIC AYA YIC CIT TYC C	(SEQIDNO: 23)
TRE-TPP-15:	TGR TCI ARI ARY TCY TTI GC	(SEQIDNO: 24)
TRE-TPP-10:	CCR TGY TCI GCI SWI ARI CC	(SEQIDNO: 25)
TRE-TPP-6:	TCR TCI GTR AAR TCR TCI CC	(SEQIDNO: 26)